Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 276 (2004) 431–440

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Wnt4 is required for proper male as well as female sexual development

Katherine Jeays-Ward[1], Mathieu Dandonneau[1], Amanda Swain*

Section of Gene Function and Regulation, Institute of Cancer Research, London SW3 6JB, United Kingdom

Received for publication 8 April 2004, revised 29 July 2004, accepted 20 August 2004
Available online 25 September 2004

Abstract

Genes previously implicated in mammalian sexual development have either a male- or female-specific role. The signaling molecule WNT4 has been shown to be important in female sexual development. Lack of *Wnt4* gives rise to masculinization of the XX gonad and we showed previously that the role of WNT4 was to inhibit endothelial and steroidogenic cell migration into the developing ovary. Here we show that *Wnt4* also has a function in the male gonad. We find that Sertoli cell differentiation is compromised in *Wnt4* mutant testes and that this defect occurs downstream of the testis-determining gene *Sry* but upstream of *Sox9* and *Dhh*, two early Sertoli cell markers. Genetic analysis shows that this phenotype is primarily due to the action of WNT4 within the early genital ridge. Analysis of different markers identifies the most striking difference in the genital ridge at early stages of its development between wild-type and *Wnt4* mutant embryos to be a significant increase of steroidogenic cells in the *Wnt4* −/− gonad. These results identify WNT4 as a new factor involved in the mammalian testis determination pathway and show that genes can have a specific but distinct role in both male and female gonad development.

Keywords: Sex determination; Wnt signaling; Gonad development

Introduction

The molecular pathways involved in mammalian sex determination and differentiation are beginning to be unraveled. Most genes that have been identified so far have been found to have a role in early testis differentiation. These include *Sry*, *Sox9*, *Dax1*, *WT1*, *PDGFRα*, *FGF9*, *Dhh*, and *GATA4* (reviewed in Jameson et al., 2003; Lovell-Badge et al., 2002; Scherer and Schmid, 2001). Testis differentiation is triggered by the action of the Y chromosome gene *Sry* in the developing gonad to determine Sertoli cell fate. One of the first Sertoli cell-specific markers to appear after the action of *Sry* in the developing XY gonad is *Sox9*, which in turn contributes to the activation of the *Amh* gene within this lineage (Arango et al., 1999; De Santa Barbara et al., 1998; Kent et al., 1996; Morais da Silva et al., 1996). Once Sertoli cells are determined, they are thought to direct the development of the rest of the testis. This process includes the formation of testicular cord and the subsequent induction of Leydig cell differentiation (reviewed in Swain and Lovell-Badge, 2002). The cell-signaling molecule Desert Hedgehog (DHH), which is produced by Sertoli cells at early stages of gonad development, has been implicated in this process (Bitgood et al., 1996; Yao et al., 2002).

Relatively few genes have been identified to be important in female sexual development. One such gene encodes the signaling molecule WNT4, which has been implicated in ovary development. *Wnt4* is expressed in the embryonic gonad of both sexes at early stages of development and after the action of *Sry* in the XY gonad its expression becomes ovary specific (Vainio et al., 1999). Lack of *Wnt4* leads to masculinization of XX embryos and it has been shown that *Wnt4* inhibits the migration of adrenal precursors and

* Corresponding author. Section of Gene Function and Regulation, Institute of Cancer Research, 237 Fulham Road, London, SW3 6JB United Kingdom. Fax: +44 207 153 5514.
E-mail address: amanda.swain@icr.ac.uk (A. Swain).
[1] These authors contributed equally to this work.

0012-1606/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2004.08.049

endothelial cells into the ovary (Jeays-Ward et al., 2003; Vainio et al., 1999). In addition, overexpression of *Wnt4* in the testis interferes with proper testicular vascular development and androgen production (Jeays-Ward et al., 2003; Jordan et al., 2003).

The nuclear hormone receptor DAX1 has been implicated in both ovary and testis development. Overexpression of *Dax1* in transgenic animals interfered with testis development, suggesting that this gene has an anti-testis function (Swain et al., 1998). Studies on a targeted mutation of *Dax1*, however, showed that *Dax1* is required for proper testis cord formation but has no obvious role in ovary development (Meeks et al., 2003; Yu et al., 1998). *Wnt4* and *Dax1* have been proposed to act in the same molecular pathway with WNT4 regulating *Dax1* expression (Jordan et al., 2001; Mizusaki et al., 2003). Consistent with this, these genes have a similar expression pattern during gonad development and *Wnt4* mutant animals show decreased levels of *Dax1* expression. In vitro studies have shown that *Dax1* transcription can be activated by β-catenin, a component of the WNT signaling pathway. These studies also showed that β-catenin acted in synergy with the nuclear hormone receptor SF1, a known activator of *Dax1* expression in the developing gonad (Mizusaki et al., 2003).

Here we show a requirement for *Wnt4* in early testis development. We find that Sertoli cell differentiation is compromised in *Wnt4* mutant testes and that this defect occurs downstream of *Sry* but upstream of *Sox9* and *Dhh*. This defect coincides with a dramatic increase of steroidogenic cells in the *Wnt4* mutant developing genital ridge.

Materials and methods

Mouse strains

The *Wnt4* mutant mice and the SF1:Wnt4 transgenic mice used were described in Jeays-Ward et al. (2003). The strain background was a mixture of 129Sv, CBA, and C57Bl6. Embryos at 11.5 and 12.5 days post coitum (dpc) were staged by counting the number of tail somites. Older embryos were staged by limb morphology.

Whole mount in situ hybridization

The conditions and probes used for whole mount in situ hybridization were described previously (Jeays-Ward et al., 2003; Swain et al., 1998; Yao et al., 2002). The whole mount in situ hybridization samples for *Cyp11a1* expression were fixed in 4% paraformaldehyde for 30 min and then dehydrated using ascending concentrations of ethanol, cleared in histoclear, embedded in paraffin, and sectioned. No difference was found between *Wnt4* +/− and +/+ embryos for all the genes analyzed at all stages.

RT-PCR

Quantitative real-time RT-PCR for *Sry* expression was performed on gonads and mesonephroi that had been dissected away from other tissue such as the developing kidney and dorsal aorta. RNA was extracted and reverse transcribed as described previously (Capel et al., 1993). The quantitative real-time PCR was done using the Quantitect SYBR Green PCR kit (Qiagen) and the ABI Prism 7700 Sequence Detector system. The PCR conditions used were an initial step of 15 min at 95°C followed by 40 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 20 s. A standard curve was constructed for every set of primers using a cDNA sample derived from a mix of various tissues. The following primers were used for *Sry* expression: 5′ GGTTGCAATCATAATTCTTCC3′ and 5′ CACTCCTCTGTGACACTTTAG3′ . Primers used for *HPRT* and *Lhx1* expression have been described previously (Capel et al., 1993; Tevosian et al., 2002). No significant relative difference in *Sry* expression between *Wnt4* +/− and −/− was found when either *HPRT* or *Lhx1* expression was used as an RNA control.

Results

Early testicular phenotype in Wnt4 mutant embryos

Detailed analysis of XY gonads from *Wnt4* mutant embryos revealed a defect in early testis development. To investigate the nature of this defect, we performed whole mount in situ hybridization on XY gonads from stage-matched mouse embryos that were homozygous (−/−), heterozygous (+/−), or wild type (+/+) for the mutant *Wnt4* allele. Analysis of expression of the Sertoli cell markers, *Sox9*, *Dhh*, and *Amh*, at the onset of their male-specific expression, 11.5 days post coitum (dpc) for *Sox9* and *Dhh* and 12 dpc for *Amh*, showed a significant reduction in Sertoli cells in *Wnt4* −/− XY gonads compared to gonads from wild-type embryos (Fig. 1A). This difference was most obvious at both ends of the gonad (at least three *Wnt4* −/− XY gonads were analyzed for each marker). No significant difference in the size of the gonad between wild type at this stage and *Wnt4* −/− embryos was observed although the shape of the gonad was slightly altered in the *Wnt4* mutant due to change in structure of the mesonephros, which lacked the Müllerian duct (Vainio et al., 1999).

Since Sertoli cell differentiation is thought to be directly regulated by *Sry* action, we therefore examined *Sry* expression in these embryos. Analysis of *Sry* expression by whole mount in situ hybridization of XY gonads from 11.5 dpc embryos showed no significant difference between wild-type and *Wnt4* mutant gonads (three *Wnt4* −/− XY gonads were analyzed) (Fig. 1B, panel a). To confirm these data, we performed quantitative real-time RT-PCR on dissected gonad and mesonephroi from stage-matched

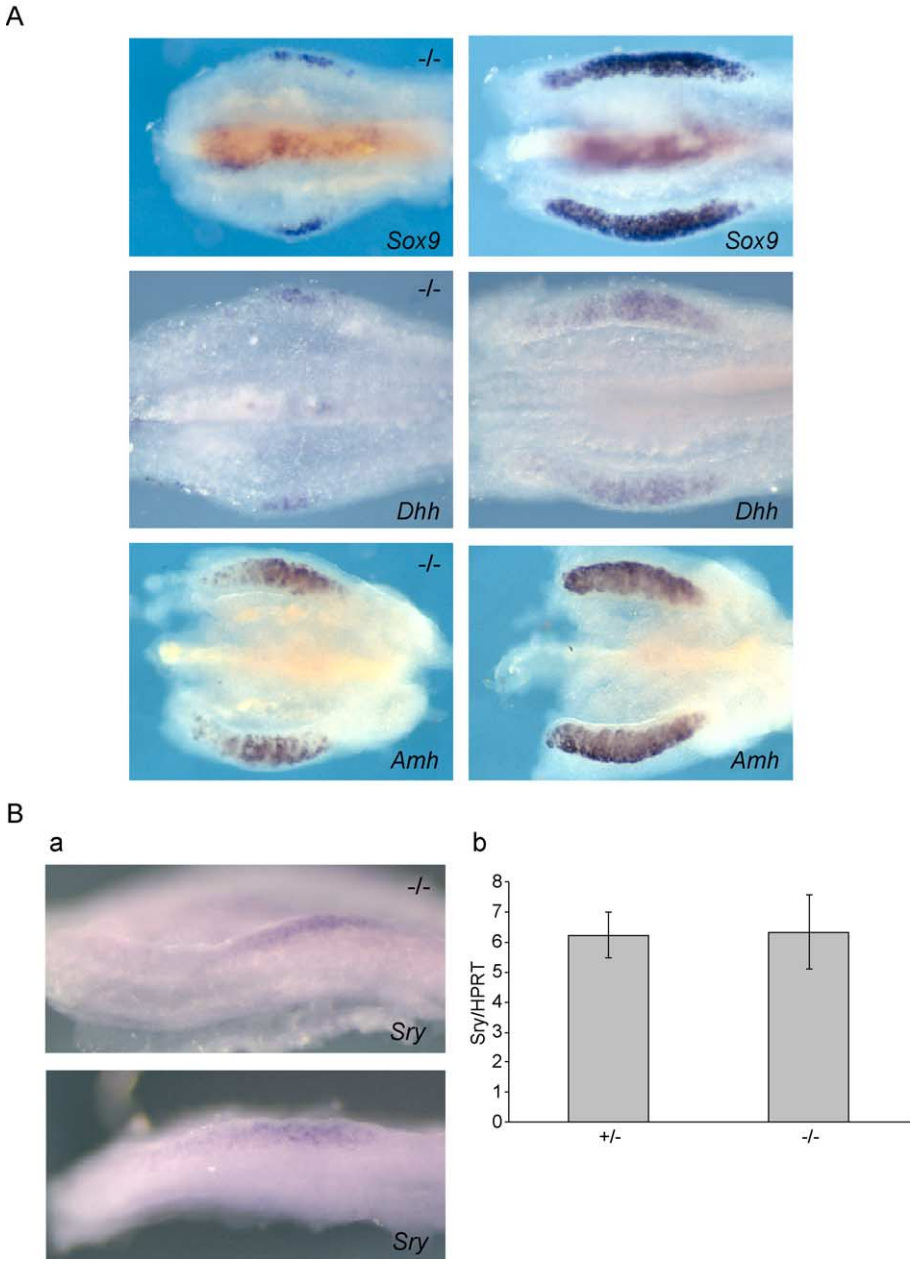

Fig. 1. (A) Sertoli cell differentiation is compromised in XY *Wnt4* mutant gonads. Whole mount in situ hybridization for *Sox9*, *Dhh*, and *Amh* expression was performed on dissected gonads, mesonephroi, and dorsal aorta region from XY *Wnt4* −/− and either +/− or +/+ embryos. The gonads were derived from 11.5 dpc embryos for *Sox9* and *Dhh* expression, whereas for *Amh* expression they were derived from 12.5 dpc embryos. Only the gonads from embryos that were homozygous for the mutant *Wnt4* allele are indicated. The anterior region of the gonad is at the left of all panels. (B) *Sry* expression is not altered in XY *Wnt4* mutant gonads. (a) Whole mount in situ hybridization for *Sry* was performed on dissected gonads and mesonephroi from XY *Wnt4* −/− and either +/− or +/+ 11.5 dpc embryos. Only gonads from embryos that were homozygous for the mutant *Wnt4* allele are indicated. The anterior region of the gonad is at the left of all panels. (b) Quantitative real-time RT-PCR was performed on dissected gonad and mesonephroi from *Wnt4* −/− and +/− XY embryos. Four samples for each genotype were analyzed. HPRT was used as an RNA standard. ±SEM values are indicated.

11.5-dpc embryos that were either heterozygous or homozygous for the mutant *Wnt4* allele. We analyzed four samples for each genotype and no significant difference in *Sry* expression was found between *Wnt4* +/− and −/− embryos (Fig. 1B, panel b). These data show that *Wnt4* contributes to Sertoli cell differentiation during testis determination and that it acts downstream of *Sry* but upstream of *Sox9* and *Dhh*.

SF1 expression in Wnt4 mutant gonads

Cell signaling by WNT4 has been proposed to regulate *Dax1* expression in the developing gonad (Jordan et al., 2001; Mizusaki et al., 2003). DAX1 was shown to be required for testis determination and differentiation (Meeks et al., 2003; Yu et al., 1998). Therefore, the decrease of *Dax1* expression in the XY *Wnt4* −/− gonad could account

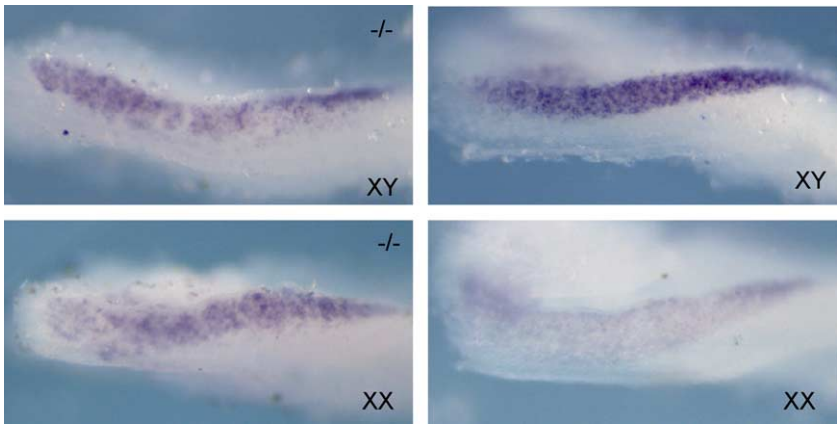

Fig. 2. Pattern of SF1 expression in *Wnt4* mutant gonads. Whole mount in situ hybridization for *SF1* expression was performed on gonads and mesonephroi from either XX or XY 11.5 dpc embryos that were −/− and either +/− or +/+ for the *Wnt4* mutant allele. Only gonads from embryos that were homozygous for the mutant *Wnt4* allele are indicated.

for the phenotype observed in these embryos. However, the testicular phenotype described here is different to that of the *Dax1* mutant mice in that we observe a defect in Sertoli cell differentiation at earlier stages whereas testes of *Dax1* mutant embryos develop normally initially and then fail to form proper testicular cords after 12.5 dpc. To further investigate this pathway, we analyzed the expression of SF1, a regulator of *Dax1* expression during early gonad development. SF1 has been shown to act in synergy with β-catenin, a downstream effector of WNT signaling, to activate *Dax1* transcription (Mizusaki et al., 2003). SF1 is expressed in the genital ridge as it first develops and after sex determination its levels are upregulated in the XY gonad, where it is expressed in Sertoli cells and Leydig cells, compared to the XX gonad (Ikeda et al., 1994). Whole mount in situ hybridization for SF1 expression at 11.5 dpc showed an unexpected difference in number of positive cells or levels of SF1 expression in −/− gonads when compared to wild-type gonads (Fig. 2). In XX embryos, there was a higher level of SF1-positive cells in the −/− gonad (three *Wnt4* −/− XX gonads were analyzed). In the XY embryo, the −/− gonad did not show the increase in SF1-positive cells seen in the wild type (three *Wnt4* −/− XY gonads were analyzed). This is not consistent with the model that the only role of WNT4 is to act with SF1 to regulate *Dax1* expression, as lack of this factor would not lead to a change in SF1 levels. Therefore, these data suggest that WNT4 has a more general role in the early gonad of both sexes.

Increase of steroidogenic cells in Wnt4 mutant gonads

Previous analysis of female *Wnt4* mutant embryos showed that *Wnt4* acted to prevent endothelial and steroidogenic cell migration from the mesonephros into the ovary (Jeays-Ward et al., 2003). We therefore investigated whether the XY *Wnt4* mutant gonad had elevated levels of endothelial or steroidogenic cell markers at earlier stages of development when sex determination was occurring. Whole mount in situ hybridization analysis of the endothelial cell marker *flk-1* showed no increase in levels in the *Wnt4* −/− gonad at 11.5 dpc when compared to wild-type or *Wnt4* +/− gonads (data not shown). In contrast, the number of cells expressing the steroidogenic marker *Cyp11a1* was found to be significantly elevated in 11.5 dpc *Wnt4* −/− gonads of both sexes when compared to wild-type gonads (at least four *Wnt4* −/− gonads were analyzed for each sex) (Fig. 3). This difference was clearly seen in sections through the gonads of wild-type and *Wnt4* mutant gonads (Fig. 3B). This difference was also observed at earlier stages of gonad development (10.5 dpc), therefore we do not think that it is due to a delay in the differentiation of the somatic cells of the *Wnt4* mutant gonad (Fig. 3A). *Cyp11a1* is normally expressed from 10.5 to 11.5 dpc in the embryonic gonad of both sexes after which it is down-regulated. This gene is then expressed in a sexually dimorphic pattern, starting at 13 dpc, where it is found in the developing Leydig cells of the testis. The *Cyp11a1* expression pattern we observed in the *Wnt4* −/− gonad of both sexes followed that of the wild-type gonad with the difference that at 10.5–11.5 dpc there were a higher number of positive cells in the mutant gonad. Our results show that *Wnt4* normally represses the increase of *Cyp11a1*-expressing cells in the early gonad of both sexes.

Ectopic WNT4 rescues early testicular phenotype in Wnt4 mutant embryos

Wnt4 is expressed in both the gonad and mesonephros at early stages of gonad development. To establish whether the defect observed was specific to WNT4 produced by the gonad and adrenal precursors, the *Wnt4* mutant animals were bred with transgenic animals carrying the *Wnt4* gene driven by the SF1 promoter (SF1:Wnt4 transgene). These transgenic animals express *Wnt4* in gonad and adrenal cells but not in the rest of the mesonephros where *Wnt4* is normally expressed (Jeays-Ward et al., 2003). Gonads from

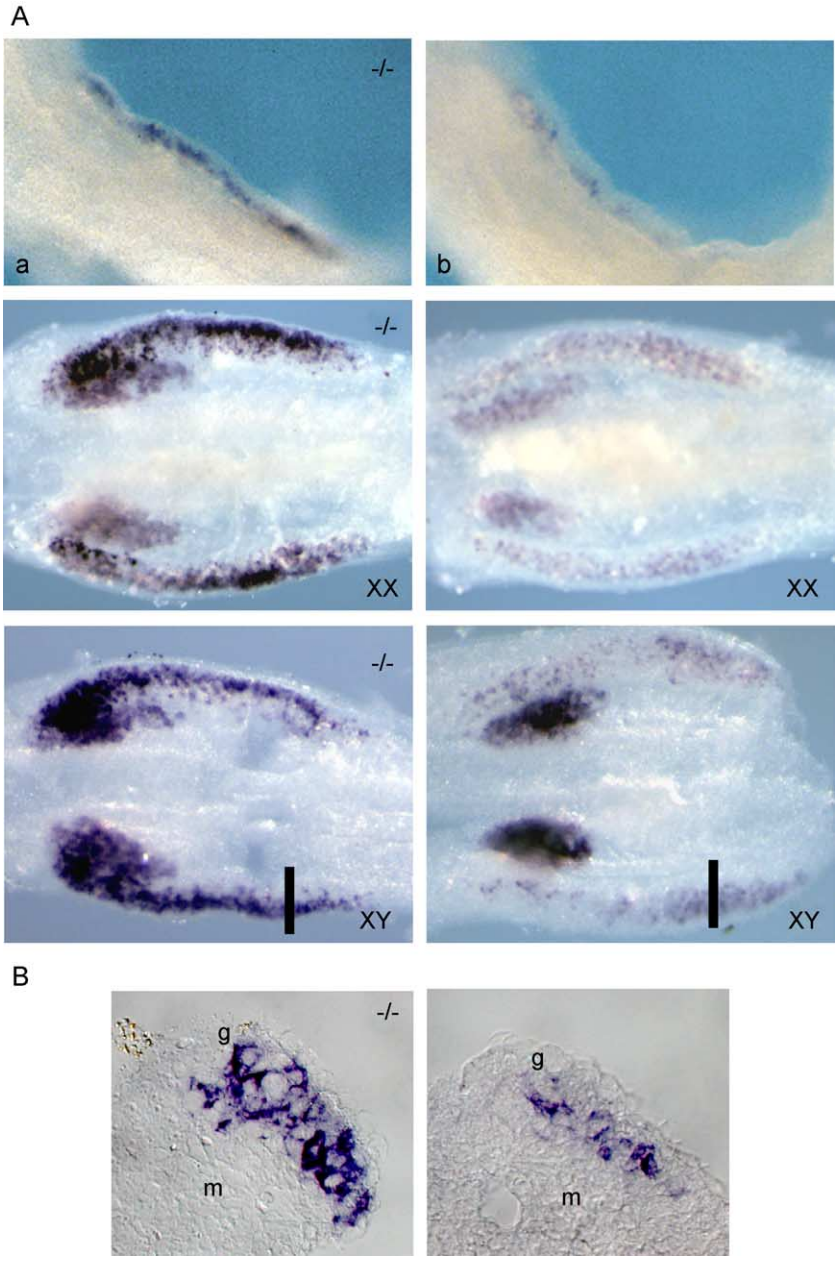

Fig. 3. Increase of steroidogenic cells in *Wnt4* mutant gonads. (A) Whole mount in situ hybridization for *Cyp11a1* expression was performed on gonads, mesonephroi, and dorsal aorta region from either XX or XY 11.5 dpc embryos that were −/− and either +/− or +/+ for the *Wnt4* mutant allele. Only gonads from embryos that were homozygous for the mutant *Wnt4* allele are indicated. The developing adrenals are also positive for *Cyp11a1*. Panels a and b show *Cyp11a1* expression in XY gonads from 10 dpc embryos. (B) Section through the posterior end of genital ridge and mesonephros from either a *Wnt4* −/− or +/− XY embryo that had been analyzed for *Cyp11a1* expression by whole mount in situ hybridization. Only gonads from embryos that were homozygous for the mutant *Wnt4* allele are indicated. Gonad (g) and mesonephros (m) are indicated. The plane of the section is indicated in panel A.

XY embryos that were mutant for the *Wnt4* allele and carried the transgene were found to lack the Sertoli cell phenotype (four *Wnt4* −/− SF:Wnt4 gonads were analyzed) and the increase in *Cyp11a1*-positive cells (three *Wnt4* −/− SF:Wnt4 gonads were analyzed) (Fig. 4).

Testis development phenotype in the Wnt4 mutant embryos

Our data show an early defect in Sertoli cell differentiation in *Wnt4* mutant embryos. To investigate the effect of this phenotype on testis development, we performed whole mount in situ hybridization for expression of Sertoli and Leydig cell markers. At 14.5 and 16.5 dpc, analysis of expression of the Sertoli cell markers, *Sox9* and *Dhh*, showed that there were fewer and more disorganized testicular cords in *Wnt4* −/− XY embryos (Fig. 5). In addition, the mutant testis was found to be smaller than wild type. Analysis of expression of the steroidogenic Leydig cell marker *Cyp11a1* at 13.5 and 14.5 dpc showed that the pattern of Leydig cells was also disorganized and overall

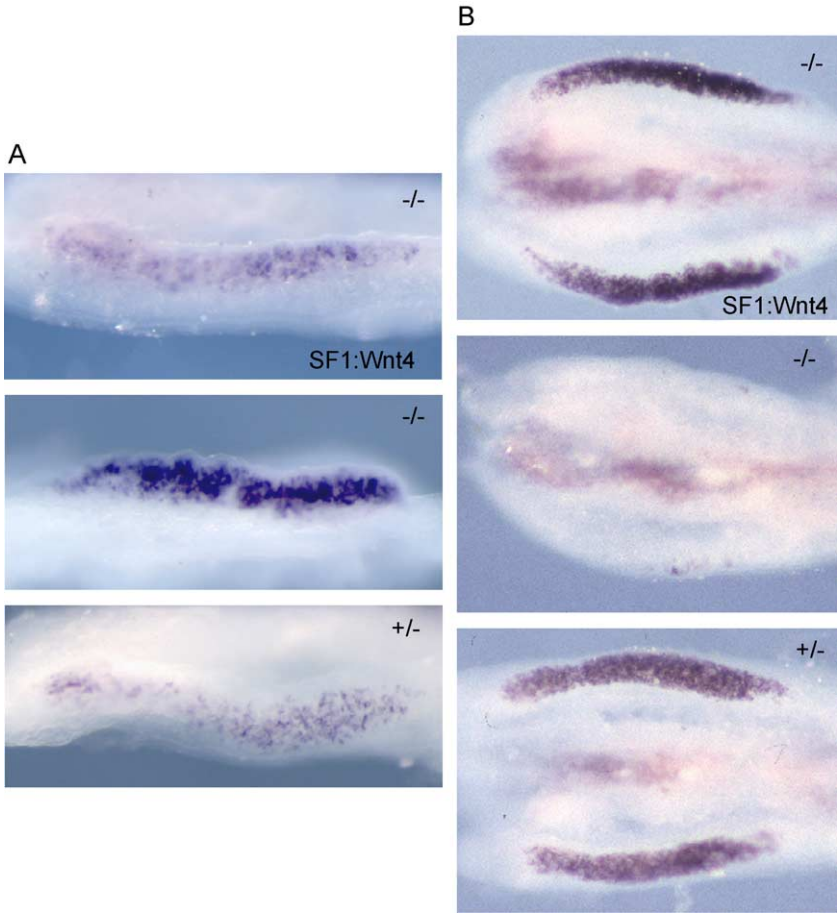

Fig. 4. Sertoli cell and steroidogenic cell phenotypes are rescued in *Wnt4* mutant XY embryos by ectopic *Wnt4* expression in the early gonad and adrenal. Whole mount in situ hybridization for *Cyp11a1* (panel A) and *Sox9* (panel B) expression was performed on 11.5 dpc gonads and mesonephroi from XY embryos that were either *Wnt4* −/− or +/− as indicated. The gonads in the top panels were derived from an embryo that contained the SF1:Wnt4 transgene.

slightly fewer Leydig cells were found. Leydig cell differentiation is dependent on proper Sertoli and testicular cord formation; therefore, this phenotype is probably a consequence of the reduction in number of testicular cords and size of the *Wnt4* −/− testis (Fig. 5). The testicular phenotype was seen in all XY gonads from *Wnt4* mutant embryos (at least four *Wnt4* −/− XY gonads were analyzed for each cell type) with varying intensity and was most noticeable at the ends of the gonad, in particular at the anterior end.

The early Sertoli cell phenotype is similar to that seen in mice carrying weak alleles of *Sry* such as the ones derived from *Mus. musculus domesticus* on a C57Bl6 background (Eicher et al., 1982; Moreno-Mendoza et al., 2004). However, this similarity is not maintained at later stages as *Wnt4* mutant animals did not develop ovotestes, although testis cord formation was affected, especially at the cortical domain just under the region where the coelomic blood vessel forms (Fig. 5). Studies on *Wnt4* and Follistatin mutant female gonads showed that the development of an ectopic coelomic blood vessel disturbed the environment of the cortical domain of the ovary and it was proposed that this could be responsible for germ cell loss in both these mutants (Yao et al., 2004). Therefore, we investigated the formation of the coelomic blood vessel in *Wnt4* mutant male gonads. Whole mount immunohistochemical analysis of the endothelial marker PECAM at 12.5 dpc showed that the testicular coelomic blood vessel in the XY *Wnt4* −/− gonad coalesced into a single vessel earlier than in the wild-type or *Wnt4* +/− embryo (Fig. 6, see Jeays-Ward et al., 2003). This suggests that the male-specific vasculature was forming earlier in *Wnt4* mutant testes. We also analyzed the expression of *PDGFRα*, a marker of the domain of the testis where the coelomic blood vessel is forming and found that this domain was increased in the XY *Wnt4* −/− gonad (Fig. 6). *PDGFRα* also marks the domain where ectopic blood vessel forms in *Wnt4* mutant female gonads (Jeays-Ward et al., 2003). Consistent with the increase in the *PDFGRα*-positive domain, whole mount in situ hybridization with the Sertoli cell markers, *Sox9* and *Dhh*, showed a reduction in the formation of testicular

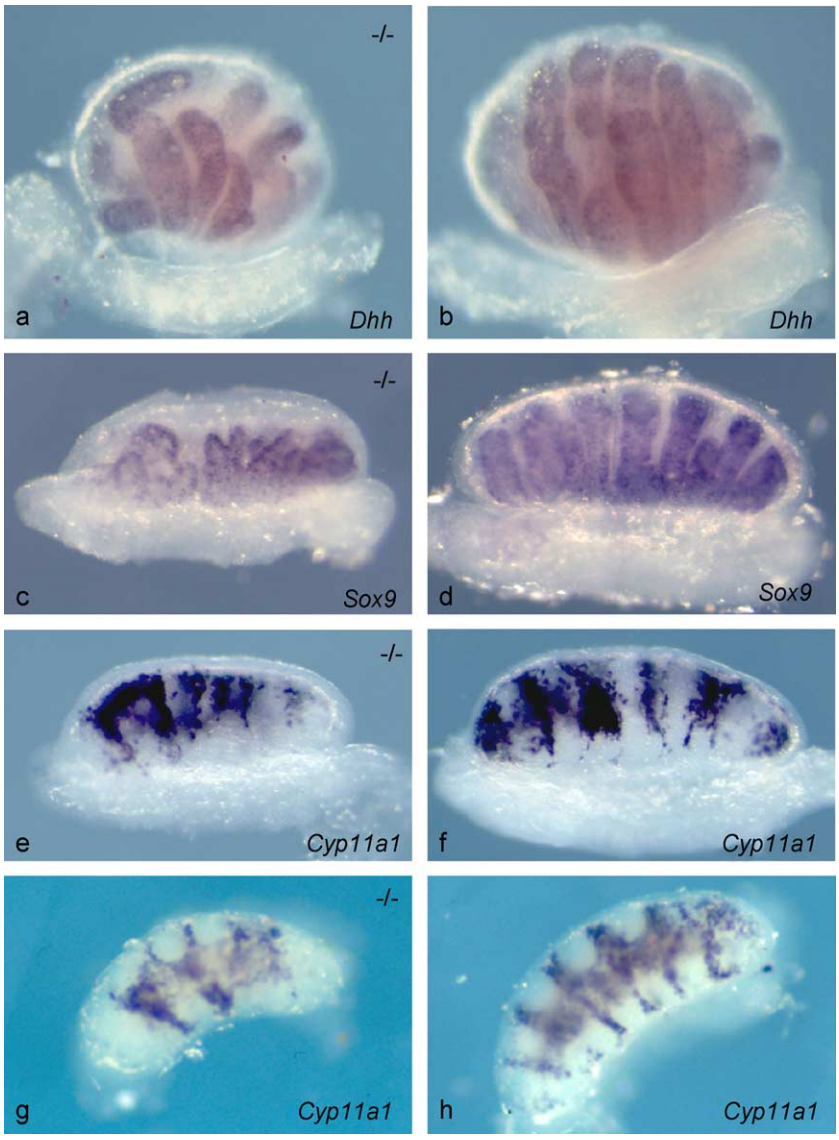

Fig. 5. Testis development is defective in *Wnt4* mutant embryos. Whole mount in situ hybridization was performed on dissected gonads and mesonephroi from 16.5 dpc (a and b), 14.5 dpc (c, d, g, and h), and 13.5 dpc (e and f) XY *Wnt4* −/− and either +/− or +/+ embryos for expression of Dhh (a and b), *Sox9* (c and d), two Sertoli cell markers, and *Cyp11a1* (e, f, g, and h), a Leydig cell marker at this stage of development. Only gonads from embryos that were homozygous for the mutant *Wnt4* allele are indicated. The images were captured at the same magnification.

cords in the cortical coelomic region in *Wnt4* mutant XY embryos at the same stage (Fig. 6).

Discussion

The data presented here show that WNT4 is required for proper testis differentiation. We find that lack of *Wnt4* gives rise to a defect in Sertoli cell differentiation at the initial stages of testis determination. This defect occurs downstream of *Sry* but upstream of *Sox9* and *Dhh*, two early Sertoli cell markers. Studies on the development of the testis indicate that if the initial step of Sertoli cell differentiation is affected, the differentiation of the rest of the testicular cell types and structures will also be affected (reviewed in Swain and Lovell-Badge, 2002). This is what we observed in the *Wnt4* mutant embryos where the testis was smaller, there were fewer testicular cords and the pattern of Leydig cell differentiation reflects that of the disorganized testicular cords.

Studies on the regulation of *Dax1* expression and the role of DAX1 in testis development suggested that the phenotype in the *Wnt4* mutant XY gonad was due to the lack of DAX1 (Jordan et al., 2001; Mizusaki et al., 2003). However, our data do not support this model. Firstly, the phenotype we observed in the *Wnt4* mutant XY gonads was different, in timing and affected cell type, to that seen in *Dax1* mutant XY embryos. Secondly, we observe a difference in the pattern of SF1 expression in the mutant gonad of both sexes at early stages of gonad development. As SF1 has been

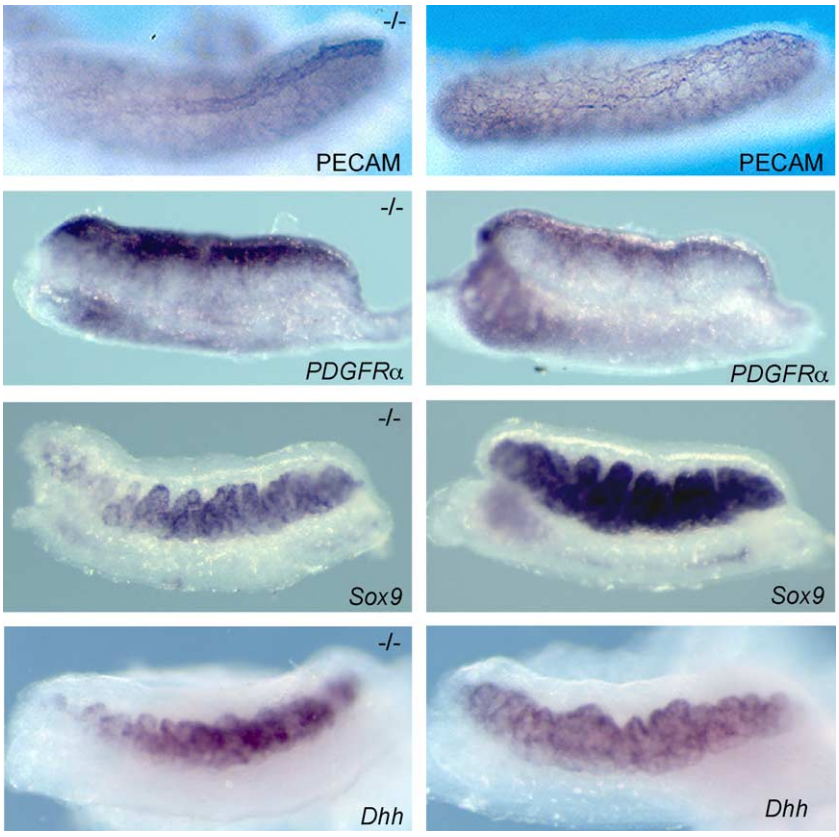

Fig. 6. Testicular coelomic vascular domain phenotype in *Wnt4* mutant embryos. Whole mount in situ hybridization for *PDGFRα*, *Sox9*, and *Dhh* expression and whole mount immunohistochemistry with an antibody to PECAM was performed on 12.5 dpc gonads and mesonephroi from XY embryos that were *Wnt4* −/− and either +/− or +/+ as indicated.

shown to act upstream of *Dax1*, a change in SF1 expression was not expected. Consistent with the decrease in Sertoli cell differentiation in the *Wnt4* −/− XY gonad, we do not observe the increase in *SF1* levels seen in wild-type XY embryos. However, we do observe an increase in *SF1* levels in the mutant gonad at early stages, especially obvious in the XX embryos, which coincides with the increase of *Cyp11a1* cells. We therefore favor the model that WNT4 has a more general role in the early gonad that contributes to proper testis development rather than it acting solely through the regulation of *Dax1* expression.

Our previous studies showed that the role of WNT4 in ovary development was to inhibit the migration of endothelial and steroidogenic cells from the mesonephros into the gonad (Jeays-Ward et al., 2003). This suggested that WNT4 was involved in cell migration at early stages of gonad development. Consistent with this, we observed an increase in steroidogenic cells in the gonad of both sexes at the time that *Sry* is acting to determine testis development in the XY gonad. Because of the small size of the gonad at these early stages, we were not able to confirm the migration hypothesis using the in vitro coculture organ culture migration assay that was used previously (Jeays-Ward et al., 2003). The rescue of the steroidogenic phenotype and the Sertoli cell defect with the SF1:Wnt4 transgenic construct shows that, as in the case of our migration studies in the ovary, it was the absence of WNT4 in the genital ridge that was responsible for both phenotypes.

The data presented here show that WNT4 acts with *Sry* to ensure proper Sertoli cell differentiation. The analysis of several markers at early stages of gonad development in the mutant embryo suggests that lack of this cell signaling molecule has a general effect on the genital ridge of both sexes. This effect has a sex-specific consequence in that it contributes to the weakening of *Sry* action in the XY gonad, probably indirectly, to determine Sertoli cell differentiation. Our expression analysis also suggests that the phenotype observed in the mutant embryo was not due to a failure of somatic cell differentiation and growth giving rise to a delay in genital ridge development. Consistent with this is the striking increase of *Cyp11a1*-expressing cells in the *Wnt4* mutant gonad of both sexes, which occurs at early stages of genital ridge development, before *Sry* action in the XY embryo. This phenotype has not been observed in other cases of sex reversal and the timing of the increase suggests that it is the primary effect of the lack of WNT4 in the genital ridge. Our studies do not show a direct link between the increase in steroidogenic cells and the Sertoli cell phenotype; however, our results are consistent with the proposal that the extra *Cyp11a1*-expressing cells in the XY mutant gonad

interfere with the action of *Sry* and prevent the proper differentiation of Sertoli cells. Cell–cell interactions are known to be important in testis determination and *Sry* may act non-cell autonomously during testis differentiation (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001). Studies with mouse chimeras derived from XX and XY embryos showed that the presence of exogenous cells, such as XX cells, within the gonad can interfere with proper testis determination (reviewed in Burgoyne and Palmer, 1993).

Absence of WNT4 in the female gonad gave rise to an ectopic coelomic blood vessel. We did not observe an increase in the level of endothelial cells at early stages of gonad development. However, we did observe a premature formation of the coelomic blood vessel in the *Wnt4* mutant testis. Our marker analysis suggests that the lack of WNT4 leads to an increase in the domain of the testis that is apportioned to vascular formation. This might lead to a decrease in the domain of the gonad where testicular cords can form and might contribute to the defect found in the testis at later stages of development. Therefore, our results suggest that the combination of the early defect in Sertoli cell differentiation and the premature formation of the coelomic blood vessel are responsible for the phenotype at later stages of testis development.

The initial study of the sexual phenotype of *Wnt4* mutant embryos did not report a phenotype in the neonatal testis (Vainio et al., 1999). Vainio et al. showed normal expression of Sertoli and Leydig cell markers such as *Amh* and *3βHSD* in the testis of neonatal mutant embryos. We find that as development proceeds, the *Wnt4* mutant testes recovered somewhat in that there were no obvious regions without testicular cords; however, we generally observe the testis to be smaller with fewer cords that are more disorganized. The difference in strain background between the two studies and the variability of the phenotype may account for this discrepancy.

The study of genes involved in gonad development has given us great insight into the process of sex determination and differentiation. The genes identified so far either have a role in the early indifferent gonad or a specific function in the ovary or testis. This work identifies a novel role for *Wnt4* in sex determination, acting with the testis-determining gene *Sry* to initiate proper testis differentiation. *Wnt4* has also been implicated in female gonad development; therefore, this study defines a new class of genes involved in sexual differentiation with a distinct function in both male and female gonad development.

Acknowledgments

We thank Alan Ashworth, Andrea Ross, and Blanche Capel for critical reading of the manuscript. AS is the recipient of a Medical Research Council Career Development Award.

References

Albrecht, K.H., Eicher, E.M., 2001. Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. Dev. Biol. 240, 92–107.

Arango, N.A., Lovell-Badge, R., Behringer, R.R., 1999. Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. Cell 99, 409–419.

Bitgood, M.J., Shen, L., McMahon, A.P., 1996. Sertoli cell signaling by Desert hedgehog regulates the male germline. Curr. Biol. 6, 298–304.

Bullejos, M., Koopman, P., 2001. Spatially dynamic expression of Sry in mouse genital ridges. Dev. Dyn. 221, 201–205.

Burgoyne, P., Palmer, S., 1993. Cellular basis of sex determination and sex reversal in mammals. In: Hillier, S.G. (Ed.), Gonadal Development and Function. Raven Press, New York, pp. 17–29.

Capel, B., Swain, A., Nicolis, S., Hacker, A., Walter, M., Koopman, P., Goodfellow, P., Lovell-Badge, R., 1993. Circular transcripts of the testis—Determining gene Sry in adult mouse testis. Cell 73, 1019–1030.

De Santa Barbara, P., Bonneaud, N., Boizet, B., Desclozeaux, M., Moniot, B., Sudbeck, P., Scherer, G., Poulat, F., Berta, P., 1998. Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene. Mol. Cell. Biol. 18, 6653–6665.

Eicher, E.M., Washburn, L.L., Whitney, J.B.D., Morrow, K.E., 1982. Mus poschiavinus Y chromosome in the C57BL/6J murine genome causes sex reversal. Science 217, 535–537.

Ikeda, Y., Shen, W.H., Ingraham, H.A., Parker, K.L., 1994. Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. Mol. Endocrinol. 8, 654–662.

Jameson, J.L., Achermann, J.C., Ozisik, G., Meeks, J.J., 2003. Battle of the sexes: new insights into genetic pathways of gonadal development. Trans. Am. Clin. Climatol. Assoc. 114, 51–63.

Jeays-Ward, K., Hoyle, C., Brennan, J., Dandonneau, M., Alldus, G., Capel, B., Swain, A., 2003. Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. Development 130, 3663–3670.

Jordan, B.K., M.M., Ching, S.T., Delot, E., Chen, X.N., Dewing, P., Swain, A., Rao, P.N., Elejalde, B.R., Vilain, E., 2001. Up-regulation of WNT-4 signaling and dosage-sensitive sex reversal in humans. Am. J. Hum. Genet. 68, 1102–1109.

Jordan, B.K., Shen, J.H., Olaso, R., Ingraham, H.A., Vilain, E., 2003. Wnt4 overexpression disrupts normal testicular vasculature and inhibits testosterone synthesis by repressing steroidogenic factor 1/beta-catenin synergy. Proc. Natl. Acad. Sci. U. S. A. 100, 10866–10871.

Kent, J., Wheatley, S.C., Andrews, J.E., Sinclair, A.H., Koopman, P., 1996. A male-specific role for SOX9 in vertebrate sex determination. Development 122, 2813–2822.

Lovell-Badge, R., Canning, C., Sekido, R., 2002. Sex-determining genes in mice: building pathways. Novartis Found. Symp. 244, 4–18.

Meeks, J.J., Weiss, J., Jameson, J.L., 2003. Dax1 is required for testis determination. Nat. Genet. 34, 32–33.

Mizusaki, H., Kawabe, K., Mukai, T., Ariyoshi, E., Kasahara, M., Yoshioka, H., Swain, A., Morohashi, K., 2003. Dax-1 (dosage-sensitive sex reversal–adrenal hypoplasia congenita critical region on the X chromosome, gene 1) gene transcription is regulated by wnt4 in the female developing gonad. Mol. Endocrinol. 17, 507–519.

Morais da Silva, S., Hacker, A., Harley, V., Goodfellow, P., Swain, A., Lovell-Badge, R., 1996. Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat. Genet. 14, 62–68.

Moreno-Mendoza, N., Torres-Maldonado, L., Chimal-Monroy, J., Harley, V., Merchant-Larios, H., 2004. Disturbed expression of Sox9 in pre-sertoli cells underlies sex-reversal in mice b6. Ytir. Biol. Reprod. 70, 114–122.

Scherer, G., Schmid, M., 2001. Genes and mechanisms in vertebrate sex determination. Introduction. Exs, XI–XII.

Swain, A., Lovell-Badge, R., 2002. Sex determination and differentiation. In: Tam, P. (Ed.), Mouse Development, vol. 17. Academic Press, pp. 371–393.

Swain, A., Narvaez, V., Burgoyne, P., Camerino, G., Lovell-Badge, R., 1998. Dax1 antagonizes Sry action in mammalian sex determination. Nature 391, 761–767.

Tevosian, S.G., Albrecht, K.H., Crispino, J.D., Fujiwara, Y., Eicher, E.M., Orkin, S.H., 2002. Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2. Development 129, 4627–4634.

Vainio, S., Heikkila, M., Kispert, A., Chin, N., McMahon, A.P., 1999. Female development in mammals is regulated by Wnt-4 signalling. Nature 397, 405–409.

Yao, H.H., Whoriskey, W., Capel, B., 2002. Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. Genes Dev. 16, 1433–1440.

Yao, H.H., Matzuk, M.M., Jorgez, C.J., Menke, D.B., Page, D.C., Swain, A., Capel, B., 2004. Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. Dev. Dyn. 230, 210–215.

Yu, R.N., Ito, M., Saunders, T.L., Camper, S.A., Jameson, J.L., 1998. Role of Ahch in gonadal development and gametogenesis. Nat. Genet. 20, 353–357.